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(54) Title: METHOD AND COMPOSITIONS FOR INDUCING BONE GROWTH (57) Abstract <p>Activin is administered systemically and locally to induce the growth of mature bone. Activin enhances the level of bone formation and the quality of the bone formed when administered locally with BMP or bone marrow. Administration of activin by subcutaneous route promotes systemic increase in the bone mass.</p>		

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5 METHOD AND COMPOSITIONS FOR INDUCING BONE GROWTHField of the Invention

The invention relates to polypeptide factors and their use in bone growth and maturation.

10 Specifically, the invention relates to the isolation and purification of activin from bone and its use in bone growth and maturation. The invention also relates to methods of (1) locally inducing mature bone growth and maturation by administering an osteogenically effective
15 amount of activin in combination with bone morphogenic proteins (BMPs) and/or bone marrow; and (2) systemically inducing bone growth and maturation by administering an osteogenically effective amount of activin alone, or in combination with BMPs and/or bone marrow.

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Background of the Invention

Activins are dimeric proteins structurally similar to inhibin, TGF- β 1, TGF- β 2, and other proteins that makeup a family of proteins structurally related to
25 TGF- β 1. These proteins exhibit the chromatographic properties of TGF- β s. In addition to having homology with respect to the amino acid sequences, activins exhibit conservation of cysteine positions characteristic of the TGF- β s. Activins exhibit a molecular weight of 25
30 kD under nonreducing conditions by SDS-PAGE (and a molecular weight of 14 kD under reducing conditions). There are two known forms of the activin subunits, which have been termed β A or β B. Homodimeric forms β AA and β BB and a heterodimeric form β AB have been described in the
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literature. Activin subunits have about a 30% homology to TGF- β 1 and TGF- β 2 chains in terms of their amino acid sequences. Inhibins are polypeptides which are also structurally related to activins. Inhibins are
5 heterodimers of the activin β A or β B subunit and a separate α subunit. Inhibins exhibit activity essentially opposite to activin.

The activin β A homodimer and β AB heterodimer have been purified from porcine follicular fluid, and
10 have been shown to stimulate the release of follicle stimulating hormone (FSH) from rat pituitary cells in vitro (W. Vale et al., Nature (1986) 321:776-79). Other reported activities include stimulation of oxytocin release from neurosecretory neurons (P.E. Sawchenko, et
15 al., Nature (1988) 334:615-17; W. Vale et al., "Recent Progress in Hormone Research" (1988) 44:1-34); stimulation of insulin secretion from pancreatic islets (Y. Totsuka et al., Biochem. & Biophys. Res. Comm. (1988) 156:335-39); and stimulation of erythroid and multi-
20 potential progenitor cell colony formation in bone marrow culture (J. Yu et al., Nature (1987) 330:765-67; H.E. Broxmeyer et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85:9052-56). Activin β A is apparently identical to erythroid differentiation factor (EDF) (M. Murata et al.,
25 Proc. Natl. Acad. Sci. U.S.A. (1988) 85:2434-38).

Despite the fact that activin is similar in amino acid sequence to TGF- β , activin does not compete with TGF- β for binding to TGF- β receptors types I, II, or III present on fibroblasts and epithelial cells.
30 However, activin has been reported to compete against binding of TGF- β 1 to rat pituitary tumor cells (S. Cheifetz et al., J. Biol. Chem. (1988) 263:17225-28). TGF- β 1 and TGF- β 2 have been reported to induce formation of endochondral bone in vivo (M.E. Joyce et al., J. Cell
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Biol. (1990) 110:2195-2207, H. Bentz, et al. (1989) J. Biol. Chem., 264:20805-10).

Although the mRNA encoding activin β A has been detected in several different tissues, including placenta, pituitary, bone marrow, kidney, spinal cord, and brain (H. Meunier et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85:247-51), to date the protein has been isolated only from porcine follicular fluid. What has not been reported is the isolation and purification of activin from bone and its ability to induce bone growth and maturation. Thus, there remains a need for the development of methodologies to extract activin from bone and to develop compositions and treatment modalities to induce bone growth and maturation. The present invention offers such.

Summary of the Invention

Bone growth and maturation are enhanced by administering activin in a formulation and in an amount sufficient for inducing bone tissue deposition. Preferably, local bone growth and maturation is induced by administering osteogenically effective amounts of a combination of activin and TGF- β , BMPs and/or bone marrow or proteins extracted therefrom. Systemic bone growth and maturation preferably is induced by administering osteogenically effective amounts of activin alone, or in combination with TGF- β , BMPs and/or bone marrow or proteins extracted therefrom. The method of the invention results in the production of bone, with little or no cartilage.

The present invention further includes compositions for locally inducing the deposition of bone tissue comprising activin with TGF- β , BMPs, and/or bone marrow or proteins extracted therefrom.

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Compositions for systemically inducing the deposition of bone tissue comprise activin alone, or in combination with TGF- β , BMPs and/or bone marrow or proteins extracted therefrom.

5 The compositions include pharmaceutical formulations, which can be administered locally and systemically, to induce bone growth and maturation, thereby effectively administered to treat bone disorders such as osteoporosis, osteomalacia, and
10 osteomalacia.

 Another object of the invention is to provide a method for locally inducing bone growth by administering activin alone or in combination with TGF- β , BMP, or bone marrow or proteins extracted therefrom, alone or in
15 combination, in a dental or orthopedic implant.

 Another object is to provide a method for extracting activin from bone and using the activin in a local or systemic treatment method to induce bone growth and maturation.

20 Another object of the invention is to provide a method for isolating and purifying activin β AA homodimer from bone tissue.

 Another feature of the invention is to regulate bone growth and maturation by first administering an
25 effective amount of TGF- β and BMPs to induce bone formation, and secondly administering an effective amount of activin to induce bone maturation.

 These and other objects, advantages and features of the present invention will become apparent to
30 those persons skilled in the art upon reading the details of the isolation, purification, formulation and use as more fully set forth below, reference being made to the accompanying drawings forming a part hereof.

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Brief Description of the Drawings

Figure 1 is a graph of dose response curves regarding the release of FSH as described in Example 2.

Figure 2 is a graph of the results obtained in an alkaline phosphatase assay at 14 days, as described in Example 3.

Figure 3 is a histological graph of the results described in Example 4.

Figure 4 is a graph of the results obtained in an alkaline phosphatase assay at 14 days, as described in Example 5.

Detailed Description of the InventionA. Overview

It must be noted that as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an activin" includes statistical mixtures of dimeric proteins of the type generally described herein; reference to "a bone morphogenetic protein" includes statistical mixtures of such proteins of the type described herein; and reference to "the method of administration" includes one or more methods of the type described herein and/or of the type which will become apparent to those of ordinary skill in the art upon reading this disclosure.

In general, the invention involves a method of inducing the deposition of mature bone tissue by administering an osteogenically effective amount of activin to a vertebrate subject in need of increased bone. The activin may be administered by itself or in combination with an additional growth factor such as TGF- β , BMP, or with bone marrow and/or proteins extracted

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therefrom. In accordance with one method of use, activin is administered (in combination with other growth factors and/or bone marrow) locally to the specific area in need of bone growth. Another method of treatment is carried out by administering an activin formulation systemically, thereby systemically inducing bone growth. In preferred embodiments, for local administration, activin in combination with BMPs and/or bone marrow, or proteins extracted therefrom, is administered in pharmaceutically acceptable excipients. For systemic administration, activin alone, or in combination with BMPs and/or bone marrow, or proteins extracted therefrom, is administered in pharmaceutically acceptable excipients.

Bone growth and maturation may be regulated by first administering an effective amount of TGF- β and BMPs in pharmaceutically acceptable excipients, to induce massive bone growth, and secondly administering an osteogenically effective amount of activin, in pharmaceutically acceptable excipients, to induce bone maturation. Alternatively, bone growth and maturation may be regulated by administering effective amounts of TGF- β , BMPs, and activin, in pharmaceutically acceptable excipients.

The activin alone or in combination with other growth factors may be administered on a daily basis for 1-14 days, and more preferably 3-10 days, and most preferably about 7 days. The amount of activin alone or in combination with other growth factors which is administered will vary depending upon the size and particular needs of the patient. Therefore, the amount administered and the frequency and length of administration will be determined by the care giver depending on the needs and the patient responsiveness with respect to the particular formulation being

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administered. In general, the ratio of BMP to activin will be about 1:0.01 to about 1:100 by weight. When activin is used in combination with bone marrow, the ratio activin to bone marrow will be about 0.001 μg : 1 g to about 100 mg : 1 g by weight.

B. Definitions

The term "activin" as used herein refers to activin βAA , activin βAB , activin βBB , and fragments thereof, synthetic peptides, and proteins having similar activity in a standard cell culture assay where the protein stimulates the release of follicular stimulating hormone (FSH) from rat pituitary cells (Vale, et al., Nature (1986) 321:776). Briefly, in this assay, anterior pituitaries from adult male Sprague-Dawley rats (200-220 g) are dissociated by collagenase and plated at a concentration of 0.33×10^6 cells per well in 24 well tissue culture dishes. The cells are allowed to recover for 72 hr in medium containing 2% fetal bovine serum (FBS). Following the recovery period, the cells are washed twice in fresh medium containing 2% FBS. All treatments are added at this time and the cells are incubated for 72 hr. The media is then collected and the FSH levels are determined using a radioimmunoassay kit provided by the National Hormone and Pituitary program of NIADDK.

For purposes of bone induction, activin may be isolated from natural sources or prepared by recombinant methods (R.H. Schwall et al., Mol. Endo. (1988) 2:1237-42). The primary sequence of activin is highly conserved from species to species (the activin βA chain is identical in human, porcine, bovine, and rat: Vale et al., Recent Progress in Hormone Research (1988) 44:1-34), as is the case with the TGF- β s. Thus, for example,

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it is expected that activin obtained from any vertebrate source will be useful in any vertebrate subject.

The term "BMPs" refers to a bone morphogenetic protein, or proteins isolated from bone, and fragments thereof and synthetic peptides which are capable of inducing bone deposition alone or when combined with appropriate cofactors such as TGF- β (or activin). Preparation of BMPs is described in PCT/US87/01537, publication number WO 88/00205, which is incorporated herein by reference to disclose BMPs -1, -2, -3, and -4, and/or their method of administration. J.M. Wozney, in Growth Factor Research, Vol. 1 (1989), pp 267-280, describes three additional BMP proteins closely related to BMP-2, and which have been designated BMP-5, -6, and -7. WO 89/09787 and 89/09788 describe a protein called "OP-1," now known to be BMP-7. The cloning of BMP-7 is described in E. Ozkaynak et al., EMBO Journal (1990) 9:2985-2093, and the purification of BMP-7 is described in T.K. Sampath et al., J. Biol. Chem. (1990) 265:13198-13205.

The BMPs may also include proteins that induce elevated levels of bone formation when combined with appropriate growth factors such as TGF- β or activin.

The term "TGF- β " refers to beta-type transforming growth factors, including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, TGF- β 5, heterodimers of the TGF- β polypeptide chains (e.g. TGF- β 1.2), and fragments thereof, synthetic peptides, and homologous proteins (having substantially equivalent biological activity in the TGF- β assay described in Methods for Preparation of Media, Supplements, and Substrate for Serum-free Animal Cell Culture (1984) pp. 181-194. Alan R. Liss, Inc.). The assay determines ability to induce anchorage-dependent growth in non-neoplastic normal rat kidney (NRK)

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fibroblasts by measuring the formation of cell colonies in soft agar. Preparation of TGF- β 1 and TGF- β 2 is described in U.S. Pat. No. 4,774,322, incorporated herein by reference. Additional TGF- β s have also been

5 described. U.S. Patent No. 4,886,747 describes the identification of TGF- β 3 and its nucleotide sequence, and describes a method for recovery of TGF- β from recombinant cell cultures. S. B. Jakowlew et al., Molec. Endocrinol. (1988) 2:1186-1195, describes TGF- β 4 and its nucleotide

10 sequence, identified by cDNA characterization. A.B. Roberts et al., Growth Factors, Vol. 2 (1990) pp. 135-147, describes the purification of TGF- β 5 from Xenopus-conditioned medium.

The term "bone growth" relates to bone mass.

15 TGF- β is thought to increase bone mass systemically. This is suggested by the increase in the number and size of osteoblasts, and increased deposition of osteoid lining bone surfaces following systemic administration.

The term "mature bone" relates to bone that is

20 mineralized, in contrast to non-mineralized bone such as osteoid. Administration of activin results in a stimulation of mature, mineralized bone formation whereas TGF- β preferentially stimulates formation of new osteoid.

The term "osteogenically effective" means that

25 amount which effects the formation and development of mature bone.

The term "subject" as used herein refers to a living vertebrate animal such as a mammal or bird in need of treatment, i.e., in need of bone induction. Such need

30 arises in cases of bone fracture, nonunion, defect, prosthesis implantation, and the like. Such need also arises in cases of systemic bone disease, as in osteoporosis.

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The term "treatment" as used herein shall mean (1) providing a subject with an amount of a substance sufficient to act prophylactically to prevent the development of a weakened and/or unhealthy state; or (2) providing a subject with a sufficient amount of a substance so as to alleviate or eliminate a disease state and/or the symptoms of a disease state, and a weakened and/or unhealthy state.

10 C. General Methods

BMPs and TGF- β s are prepared by methods known in the art (see e.g., PCT/US87/01537 and 4,774,322 which are incorporated herein by reference to disclose such), or are available from commercial sources (R&D Systems, Minneapolis, MN). Activin may be isolated from follicular fluid, prepared by recombinant methods, or isolated from bone using the methods disclosed below.

Activin is similar in hydrophobicity to TGF- β 1 and TGF- β 2 (but has a lower pI than these polypeptide factors) and is isolated from bone in a similar fashion. J. M. Vaughan et al., Meth. Enzymol. (1989) 168:588-617, describes a method for preparing anti-activin β A antibody. It is well known in the art that antibodies are useful in isolation and purification procedures. Alternatively, a detailed isolation procedure in accordance with the present invention is described in Example 1 below. The characterization of activin is described in Example 2 below.

Pharmaceutical formulations of the invention which include activin for administration will generally include an osteogenically effective amount of activin, and optionally, BMP, to promote bone growth, in addition to a pharmaceutically acceptable excipient. Suitable excipients include most carriers approved for parenteral

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administration, including water, saline, Ringer's solution, Hank's solution, and solutions of glucose, lactose, dextrose, ethanol, glycerol, albumin, and the like. These compositions may optionally include

5 stabilizers, antioxidants, antimicrobials, preservatives, buffering agents, surfactants, and other accessory additives. A presently preferred vehicle comprises about 1 mg/ml serum albumin (species-specific) in phosphate-buffered saline (PBS). A thorough discussion of suitable

10 vehicles for parenteral administration may be found in E.W. Martin, "Remington's Pharmaceutical Sciences" (Mack Pub. Co., current edition sections relating to the excipient vehicles and formulating being incorporated herein by reference to disclose such). Such formulations

15 are generally known to those skilled in the art and are administered systemically to provide systemic treatment.

Compositions of the invention may also be implanted directly at the site to be treated, for example, by injection or surgical implantation of the

20 composition in a sustained-release carrier. Suitable carriers include hydrogels, controlled- or sustained-release devices (e.g., an Alzet® minipump), polylactic acid, and collagen matrices. Presently preferred carriers are formulations of atelopeptide collagen

25 containing particulate calcium phosphate mineral components, such as combinations of fibrillar atelopeptide collagen (for example Zyderm® Collagen Implant, available from Collagen Corporation, Palo Alto, California) with hydroxyapatite-tricalcium phosphate (HA-

30 TCP, available from Zimmer, Inc., Warsaw, IN). It is presently preferred to administer implant compositions containing activin and BMP in a collagen/mineral mixture implant.

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The proteins of the invention may be conjugated to other molecules to increase their water-solubility, increase their half-lives, or enhance their ability to bind to bone. For instance, they may be conjugated to polyethylene glycol to increase their water solubility or to bone-binding molecules such as bisphosphonates (e.g. 1-hydroxyethylidene-1,1-bisphosphonic acid, dichloromethylene bisphosphonic acid, and 3-amino-1-hydroxypropylidene-1-bisphosphonic acid) and fluorochromes (e.g. tetracyclines, calcein blue, xylenol orange, calcein green, and alizarin complexone red) to target the proteins to bony sites. Various agents for conjugating molecules to proteins are well known in the art and include aldehydes, carbodiimides, and other bifunctional moieties.

The precise dosage necessary will vary with the age, size, sex and condition of the subject, the nature and severity of the disorder to be treated, and the like; thus, a precise effective amount cannot be specified in advance and will be determined by the care giver. However, appropriate amounts may be determined by routine experimentation with animal models, as described below. In general terms, an effective dose of activin for systemic treatment will range from about 0.001 $\mu\text{g/kg}$ to about 10 mg/kg of body weight. An effective dose for BMP is about 0.001 $\mu\text{g/kg}$ to about 10 mg/kg of body weight.

An effective dose for activin for local treatment will range from about 0.001 $\mu\text{g/kg}$ to about 10 mg/kg of body weight. An effective dose of BMP for local treatment is substantially the same as the dose of activin.

In addition, it may be desirable to combine the proteins with other therapeutics, such as, for instance in the case of osteoporosis, fluoride, calcitonin,

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vitamin D metabolites, estrogen, and parathyroid hormone. Because proteins are non-species-specific in their activity they maybe used to treat subjects in general, including sport, pet, and farm animals, and humans.

5 Suitable animal models for bone growth and maturation include the mouse model as illustrated in Examples 3, 4 and 5 below. Briefly, systemic treatment is assayed by injecting test amounts of activin into a mouse or other experimental animal following a standard
10 protocol, followed by examination of bone strength and mass at approximately 14 days post-administration. Such examination may be performed *in situ* by using imaging techniques (e.g., nuclear magnetic resonance imaging, X-ray tomography, ultrasound, and sound conduction) or
15 stress testing, or *ex vivo* by standard histological methods. Suitable dosages and protocols will result in improved deposition of mature bone, for example, increased cortical thickness in long bones.

 Compositions of the invention are useful for
20 treating bone fractures, defects, and disorders which result in weakened bones such as osteoporosis, osteomalacia, and age-related loss of bone mass. Compositions of the invention that are delivered in sustained-release vehicles are also partic-
25 ularly useful for improving implant fixation, for example for improving ingrowth of new bone into a metal prosthesis in joint reconstruction and dental or orthopedic implants.

 Dental and orthopedic implants can be coated
30 with osteoinductive proteins, such as BMP, activin, TGF- β , and bone marrow or proteins extracted therefrom, either in combination or alone, to enhance attachment of the implant device to the bone. Implant devices
35 generally have a porous end, which is inserted into the

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implant site. The porous end allows the new bone to grow into the porous space and hold the implant in place. The greater the growth of new bone into the porous space, more solidly the implant is held in place. The presence of osteoinductive proteins, such as BMP, activin, and TGF- β , and bone marrow or proteins extracted therefrom, either alone or in combination, in the porous space is expected to enhance the growth of new bone into the porous space.

In general, implant devices may be coated with osteoinductive proteins as follows. Activin, BMP, and TGF- β , and bone marrow or proteins extracted therefrom, either alone or in combination, are dissolved at a concentration in the range of 0.01 μ g/ml to 200 mg/ml in phosphate-buffered saline (PBS) containing 2 mg/ml serum albumin. The porous end of an implant is dipped in the solution and is airdried or implanted immediately into the bony site. The viscosity of the coating solution is increased, if desired, by adding hyaluronate at a final concentration of 0.1 mg/ml to 100 mg/ml or by adding other pharmaceutically acceptable excipients. Alternatively, the solution containing the osteoinductive factor is mixed with collagen gel (e.g. Zyderm® Collagen Implant, Collagen Corp., Palo Alto, CA) to a final collagen concentration of 5 mg/ml to 100 mg/ml to form a paste, which is then used to coat the porous end of the implant device. The coated implant device is placed into the bony site immediately or is airdried and rehydrated with PBS prior to implanting.

Either native or synthetic (recombinant) activin can be used to produce antibodies, both polyclonal and monoclonal. The term "antibody" is intended to include whole immunoglobulin of any isotype or species as well as antigen binding fragments and

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chimeric constructs. If polyclonal antibodies are desired, purified activin is used to immunize a selected mammal (e.g. mouse, rabbit, goat, horse, etc.) and serum from the immunized animal later collected and treated according to known procedures. Compositions containing polyclonal antibodies to a variety of antigens in addition to activin can be made substantially free of antibodies which are not anti-activin antibodies by passing the composition through a column to which activin has been bound. After washing, polyclonal antibodies are eluted from the column. Monoclonal anti-activin antibodies can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus, M. Schrier, Hybridoma techniques (1980); Hammerling et al., Monoclonal Antibodies and T cell Hybridomas (1981); Kennett et al., Monoclonal Antibodies (1980).

By employing activin (native or synthetic) as an antigen in the immunization of the source of the B cells immortalized for the production of monoclonal antibodies, a panel of monoclonal antibodies recognizing epitopes at different sites on the activin molecule can be obtained. Antibodies which recognize an epitope in the binding region of the protein can be readily identified in competition assays between antibodies and proteins. Antibodies which recognize a site on the activin protein are useful, for example, in the purification of the protein from cell lysates or fermentation media, in the characterization of the protein, and in identifying immunologically related

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proteins. Such immunologically related proteins (i.e. that exhibit common epitopes with activin) are another aspect of the invention. In general, as it is known in the art, the anti-activin antibody is fixed (immobilized) to a solid support, such as a column or latex beads, contacted with a solution containing activin, and separated from the solution. The activin, bound to the immobilized antibodies, is then eluted.

Methods for inducing local bone growth and maturation comprise administering an osteogenically effective amount of activin, in combination with BMPs, bone marrow, or proteins extracted therefrom, in a pharmaceutically acceptable carrier, as describe in Example 3, below.

Methods for inducing systemic bone growth and maturation comprises administering an osteogenically effective amount of activin alone, and optionally including BMPs, bone arrow, or proteins extracted therefrom, in a pharmaceutically acceptable carrier, as described in Example 4, below.

Methods for regulating bone growth and maturation comprises initially administering an effective amount of TGF- β and BMPs, and sequentially administering an effective amount of activin. Preferably, to induce endochondral bone, combinations of BMP and TGF- β may be locally administered to induce cartilage model. Activin may be subsequently administered subcutaneously and/or systemically to induce mature bone formation and differentiation of the cartilage model induced by TGF- β and BMP. The activin would enhance mineralization of the endochondral bone and maturation. It is expected that the quantity of mature bone formed by this method would be greater than the quantity of mature bone formed by either (i) TGF- β , BMPs, or activin alone, or (ii)

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combinations of TGF- β and BMPs, or (iii) combinations of activin and BMPs.

Alternative methods for regulating bone growth and maturation comprise (i) locally administering an
5 osteogenically effective amount of activin with TGF- β , BMP and/or bone marrow or proteins extracted therefrom, to induce bone growth and maturation, or (ii) systemically administering effective amounts of TGF- β , BMP and/or bone marrow or proteins extracted therefrom to
10 induce bone growth, followed by systemically administering an osteogenically effective amount of activin to induce bone maturation.

Activin is extracted from demineralized bone powder with 6M guanidine HCl, 10 mM EDTA, pH 6.8. The
15 extract is fractionated by gel filtration chromatography. The pool of fractions containing TGF- β is desalted and fractionated by cation exchange chromatography. The pool of fractions containing activin is then fractionated by C18 RP-HPLC, using a linear acetonitrile gradient in 0.1%
20 trifluoroacetic acid (TFA). Activin elutes between TGF- β 1 and TGF- β 2. The activin fraction is applied onto a cation exchange Mono-S FPLC column (Pharmacia) at pH 4.6. The column is equilibrated sequentially into pH 6.7 and pH 9.0 buffers. Activin, which elutes during the pH 6.7-
25 9.0 gradient, is desalted by C18 RP-HPLC performed in 0.1% TFA with a linear acetonitrile gradient as the final purification step.

Since TGF- β 1 and TGF- β 2 have similar chromatographic properties as activin, activin
30 preparations may be contaminated with low levels of TGF- β . The majority of the contaminating TGF- β activity may be removed during the pH 4.6-6.7 gradient performed on the Mono-S FPLC, resulting in substantially pure preparations of activin. In addition, the majority of
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TGF- β 2 elutes slightly later than activin during the pH 6.7-9.0 gradient, while TGF- β 1 is retained on the column during the Mono-S FLPC step. The use of a two step salt gradient, as compared to standard techniques using a single step salt gradient, is a unique chromatographic procedure which results in significantly pure preparations of activin.

D. Examples

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to extract, isolate, formulate and use the compositions and methods of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to insure accuracy with respect to numbers used (e.g., amounts, times, temperature, etc.), but some experimental error and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in degrees centigrade, pressure is at or near atmospheric, and other parameters are conventional and in accordance with those normally accepted by those skilled in the art.

Example 1

(Extraction and Isolation of Activin)

Activin β AA was isolated and purified from 75 kg of bovine bone powder using the procedure described below. The extract of demineralized bone powder was prepared from fresh metatarsal and metacarpal bones as described by Seyedin et al., U. S. Patent Number 4,774,322.

Crude bone extract in 6 M guanidine HCl, 10 mM EDTA, pH 6.8, was fractionated by column chromatography

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on Sephacryl® S-200, and the fractions containing high levels of TGF- β were pooled. The TGF- β pool was concentrated by ultrafiltration and was desalted by passage through an Amicon GH-25 column equilibrated in 6 M urea, 50 mM sodium acetate, 10 mM NaCl, pH 4.6.

The NaCl concentration of the TGF- β pool was increased to 70 mM, and the product applied onto a Whatman® CM-52 column (2.5 × 38 cm) equilibrated in a buffer of 6 M urea, 50 mM sodium acetate, 70 mM NaCl, 1% isopropanol, pH 4.6. The urea/acetate buffer was pumped through the column until A_{280} returned to the baseline. The bound protein was eluted using a linear 70-600 mM NaCl gradient (1000 ml total volume) in the urea/acetate buffer at a flow rate of 30 ml/hour. The CM-52 fractions were analyzed by SDS-PAGE (U. Laemmli, Nature (1970) 227: 680-85) on a 15% polyacrylamide gel, and three separate CM-bound pools were made. Pools 2 (fractions 61-70) and 3 (fractions 71-83) contained the majority of TGF- β 2 and TGF- β 1, respectively. Pool 1, which consisted of fractions 55-60, contained the majority of activin.

Each of the pools was individually chromatographed on a C18 RP-HPLC column (1 × 25 cm, Vydac, 218TP510) equilibrated in 0.1% trifluoroacetic acid (TFA). The bound protein was eluted from the column using a linear gradient of acetonitrile from 32-52% buffer B (90% acetonitrile, 0.1% TFA) over 20 minutes at a flow rate of 3 ml/minute. Fractions were collected manually in order to isolate individual peaks, and the fractions were analyzed by SDS-PAGE under nonreducing conditions. A 25 kD protein (activin) migrating slightly faster than TGF- β on an SDS-polyacrylamide gel was observed to elute from the reverse-phase HPLC column between the elution positions of TGF- β 1 and TGF- β 2 in the CM-bound pools 1 and 2. The CM-bound pool 3, which

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contained the majority of the TGF- β 1, was later shown to contain very little activin, and no further attempts were made to isolate it from pool 3.

The 25 kD protein (activin) fractions from the CM-bound pools 1 and 2 were combined. The pool was diluted 3-fold with 6 M urea, 50 mM sodium acetate, 10 mM NaCl, 1% isopropanol, pH 4.6, and was loaded onto a Mono-S FPLC column (Pharmacia HR5/5), which was equilibrated in the same buffer. Some of the bound proteins eluted when the pH was raised from 4.5 to 6.7 by equilibrating the column into 6 M urea, 50 mM sodium acetate, 10 mM NaCl, 1% isopropanol, pH 6.7 at a flow rate of 0.5 ml/min over a 10 minute period. The column was then equilibrated into 6 M urea, 20 mM HEPES, 10 mM NaCl, 1% isopropanol, pH 6.7, and the 25 kD protein (activin) was eluted by equilibrating the column into 6 mM urea, 20 mM HEPES, 10 mM NaCl, 1% isopropanol, pH 9.0, at a flow rate of 0.5 ml/min over a 10 min period.

A linear NaCl gradient from 10-400 mM NaCl in urea/HEPES buffer, pH 9.0, at a flow rate of 0.5 ml/min over a 10 min period, was then used to elute remaining bound proteins. The SDS-polyacrylamide gels were stained with silver (J.H. Morrissey, Anal. Biochem. (1981) 117:301-310). The majority of TGF- β activity, which would otherwise contaminate activin, was removed by performing the pH 4.7 - 6.7 gradient. Activin eluted during the pH 6.7 - 9.0 gradient. The elution position of activin is slightly before that of TGF- β 2 during the pH 6.7 - 9.0 gradient. TGF- β 1 elutes during the 10 - 400 mM NaCl gradient at pH 9.0.

Some 25 kD TGF- β -like protein (including TGF- β 1) eluted during the NaCl gradient and was highly contaminated with low molecular weight proteins (12 kD to 18 kD).

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The 25 kD protein (activin) was chromatographed on a C18 RP-HPLC column as the final purification step. The pool of 25 kD protein fractions from the pH 6.7 - 9.0 gradient of the Mono-S chromatography was
5 acidified by adding glacial acetic acid to a final concentration of 1.0 M, and was applied to a C18 RP-HPLC analytical column (Vydac, 4.6 × 250 mm, 218TP54) equilibrated in 0.1% TFA. The column was run at a flow rate of 1 ml/min, and the bound protein was eluted using
10 a linear acetonitrile gradient from 32-52% buffer B at a rate of 1% buffer B/min. Fractions were analyzed by SDS-PAGE under nonreduced conditions. The activin fraction demonstrated one single major band on the silver-stained SDS-polyacrylamide gel. No other bands were seen. The
15 total yield of the 25 kD protein (activin) from the CM-bound pools 1 and 2 was approximately 1.2 mg, corresponding to 16 µg/kg bone powder.

Example 2

20 (Identification of the 25 kD Protein as Activin)

(A) The N-terminus of the 25 kD protein obtained in Example 1 above was sequenced on an Applied Biosystems sequencer. The first 36 amino acids were Gly-Leu-Glu-(?)-Asp-Gly-Lys-Val-Asn-Ile-(?)-(?)-Lys-Lys-Gln-
25 Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Glu-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro-Ser-Gly-Tyr-His (where "?" denotes an unidentified amino acid). This sequence corresponds to the first 36 amino acid residues at the N-terminus of the activin βA subunit, in which each of the "?"s is Cys
30 (Cys is not detected in the sequencing process). No other amino acid sequences were observed. The entire activin βA subunit sequence, in single letter code, is

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GLECDGKVNICCKKQFFVSF
KDIGWNDWIIAPSGYHANYC
EGECPSHIAGTSGSSLSFHS
TVINHYRMRGHSPFANLKSC
CVPTKLRPMSMLYYDDGQNI
IKKDIQNMIVEECGCS

(W. Vale et al., Recent Progress in Hormone Research
(1988) 44:1-34.

(B) A 5 μ g sample of the activin obtained in
Example 1 above was reduced and denatured by incubating
for 2 hours at 50°C under N₂ atmosphere in 250 μ L of
alkylation buffer (0.4 M Tris/HCl, pH 8.5, 6 M urea, 0.1%
EDTA, 20 mM DTT). The reduced protein was alkylated by
incubating in the presence of 100 mM iodoacetamide at
room temperature in the dark for 4 hrs. The sample was
subjected to SDS-PAGE.

The silver-stained gel of reduced and alkylated
25 kD protein exhibited a single band migrating at a
molecular weight of 14 kD. This observation is consistent
with the 25 kD protein being activin, where activin is
known to be a dimer of two 14 kD subunits.

(C) The activin extracted and isolated in
Example 1 above was assayed for biological activity by
testing the ability to stimulate release of follicle-
stimulating hormone (FSH) by rat pituitary cells in
culture following the method of W. Vale et al.,
Endocrinology (1972) 91:562-572. Briefly, anterior
pituitaries from adult male Sprague-Dawley rats (200-220
g) were dissociated by collagenase and plated at a
concentration of 0.33×10^6 cells per well in 24-well
tissue culture dishes. The cells were allowed to recover
for 72 hours in medium containing 2% fetal bovine serum
(FBS). Following the recovery period, the cells were
washed twice in medium containing 2% FBS. All treatments

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were added at this time and the cells were incubated for 72 hr. The media was then collected and FSH levels determined using a radioimmunoassay kit provided by the National Hormone and Pituitary Program of NIADDK. The protein isolated in Example 1 exhibited FSH-releasing activity equal to activin derived from porcine follicular fluid. TGF- β 1 and TGF- β 2 did not exhibit FSH-releasing activity, as expected. The results are shown in Figure 1. The results described in (A), (B) and (C) demonstrate that the 25 kD protein purified from bone in Example 1 is activin BA homodimer.

The activin purified from bovine bone, as described in Example I, was assayed for the protein's ability to stimulate formation of erythroid colonies following the method of Broxmeyer et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85:9052-6. Single cell suspensions of normal C57B1/6 male murine femur bone marrow and spleen were prepared for in vitro assay. Activin stimulated increases in the colony-forming unit erythroids (CFU-E) and burst-forming unit erythroid (BFU-E) in a dose-dependent fashion in the similar range of activin concentrations as those reported for recombinant human activin (Broxmeyer et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85:9052-6). Activin also significantly stimulated both the CFU-E and BFU-E of spleen cells. The results are summarized in Table I below.

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Table One: Effects of activin on erythroid colony formation

Bone marrow (BM)

GROUP	CFU-E/ 10^5 BM	%CONTROL	BFU-E/ 10^5 BM	%CONTROL
Control	215 \pm 38		100 10 ± 2	100
Activin 100 ng/ml	309 \pm 32 **		143 19 ± 1 **	188
50 ng/ml	296 \pm 15		138 16 ± 2 *	163
25 ng/ml	269 \pm 25		125 16 ± 4 *	155
12.5 ng/ml	245 \pm 51		114 15 ± 4	150
6.25 ng/ml	239 \pm 22		111 13 ± 3	125
3.12 ng/ml	229 \pm 20		106 10 ± 2	100
1.56 ng/ml	215 \pm 34		100 10 ± 2	100
Spleen	BFU-E/ 10^5	% control	CFU-E/ 10^5	% control
control	2.4 + 0.4	100	255 + 16	100
50 ng/ml activin	7 + 1	271	429 + 20	168

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Example 3

(Local Induction of Bone Growth and Maturation)

The following experiment demonstrated induction of bone growth *in vivo* using a combination of BMP and activin.

A ceramic/collagen carrier was prepared using hydroxyapatite, tricalcium phosphate, and soluble bovine dermal collagen (VITROGEN® collagen-in-solution, Collagen Corporation) as described by H. Bentz et al., J. Biol. Chem. (1989) 264:20805-810. BMP was prepared as described in E.A. Wang et al., Proc. Natl. Acad. Sci. USA (1988) 85:9484-88. Activin was obtained as described in Example 1 above.

Compositions were prepared as follows:

- 15 A: Carrier alone
- 1: BMP (1 μ g)
- 2: BMP (1 μ g), TGF- β 2 (7.5 μ g)
- 3: BMP (1 μ g), TGF- β 2 (2.5 μ g)
- 4: BMP (1 μ g), activin (7.5 μ g)
- 20 5: BMP (1 μ g), activin (2.5 μ g)
- 6: BMP (1 μ g) + TGF- β 2 (7.5 μ g)
- + activin (7.5 μ g).
- 7: activin (2.5 μ g)

The compositions were then implanted at the ectopic subcutaneous sites in the ventral thorax region of male Sprague-Dawley rats at 6 rats/composition (except for carrier alone, BMP alone, and activin alone, at 4 rats/composition. After 14 days, the implants were excised, evaluated biochemically for alkaline phosphatase activity (Bentz et al., J. Biol. Chem. (1989) 264:20805-20810; Reddi et al., Proc. Natl. Acad. Sci. U.S.A. (1972) 69:1601-5) and examined histologically for bone growth. The biochemical results are shown graphically in Figure 2.

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Biochemistry:

Implants containing BMP or activin alone exhibited very low alkaline phosphatase activity. (See columns 1 and 7 in Figure 2.) The compositions containing BMP and either TGF- β 2 or activin or both (at either dosage) exhibited high levels of alkaline phosphatase activity.

10 Histology:

Compositions containing carrier alone, or carrier with activin, exhibited no bone growth, and displayed mild to moderate foreign body reactions. Compositions containing BMP alone exhibited low to moderate levels of bone and no cartilage.

Compositions containing BMP and TGF- β 2 exhibited a large increase in both bone and cartilage. Composition 3 (2.5 μ g TGF- β 2) exhibited more bone, and more differentiation of the bone tissue, than composition 2 (7.5 μ g TGF- β 2). Composition 2 induced more cartilage than bone.

Compositions containing BMP and activin (4 and 5) exhibited little or no cartilage, and significantly elevated levels of deposited mature bone. Composition 5 (2.5 μ g activin) induced deposition of more bone than composition 6 (7.5 μ g activin). However, both compositions induced very little cartilage deposition, and produced more mature bone than that obtained using BMP with TGF- β 2.

Composition 6 (BMP, TGF- β 2, and activin) induced a poorly differentiated, disorganized mixture of bone, fibrocartilage, and fibrosis.

The experimental results demonstrated that activin functions as an osteogenic cofactor for BMP in

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vivo, and induces growth of high-quality mature bone, without inducing cartilage formation.

Example 4

5 (Systemic Administration of Activin)

The following experiment demonstrated the activity of activin upon systemic administration in vivo.

Neonatal Mice:

10 Neonatal mice (Swiss-Webster, 8-10 litters per group) were given daily administrations of:

- 1: 1 μ g TGF- β 1
- 2: 1 μ g activin
- 3: 3 μ g activin
- 15 4: 1 μ g TGF- β 1 + 1 μ g activin
- 5: vehicle alone

in a vehicle of 37.5 μ g mouse serum albumin (MSA) in 25 μ l PBS. The control group received vehicle alone. The compositions were administered daily for 4 days, by subcutaneous injection in the nape of the neck. The femurs were harvested on the fifth day, and examined histologically.

20 The mice receiving 1 μ g/day activin exhibited superior bone growth and maturation, displaying reduced porosity in the femoral shaft compared with controls. In contrast, mice receiving 1 μ g/day TGF- β 1 exhibited increased porosity compared with controls. Both groups exhibited increased levels of osteoclasts, and hypertrophy of osteoblasts relative to controls, suggesting increased bone remodeling. However, the group receiving 30 3 μ g/day activin exhibited decreased levels of osteoclasts, and flat, inactive-appearing osteoblasts. Administration of activin and TGF- β 1 together inhibited the changes in osteoclasts and osteoblasts, and did not

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increase femoral shaft porosity. The results are graphically shown in Figure 3.

The results indicate that systemic administration of activin for a limited treatment course improves bone growth and maturation by inducing bone formation and promoting bone remodeling.

Example 5

(Activin Enhances Osteogenesis in Bone Marrow)

Implantation of bone marrow in collagen/hydroxyapatite-tricalcium phosphate ceramic carrier in rat subcutis results in local bone formation in the ceramic implant at ectopic sites. This process, known as osteogenesis, is probably due to differentiation of a specific cell population in the bone marrow. Activin, when added to the bone marrow, promotes greater mature bone deposition.

Young adult male Lewis rats (8 weeks old, 3 rats/group) were implanted subcutaneously in the ventral thorax region with ceramic carrier containing 25 or 50 μ l of rat bone marrow plus 5 μ g of activin or TGF- β 2. After 14 days, the implants were excised, evaluated for alkaline phosphatase activity and examined histologically for bone formation.

Ceramic carrier alone had almost no alkaline phosphatase activity and histologically, exhibited low levels of inflammatory cells and no bone. Ceramic carrier with 20 μ l of bone marrow exhibited low levels of bone formation. However, the group with 20 μ l of bone marrow plus activin showed elevated levels of bone formation over the levels observed with 20 μ l of bone marrow alone. Alkaline phosphatase activity was elevated in the implants of bone marrow alone and bone marrow plus activin when compared with the carrier alone (Figure 4).

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In a second study, 10 μ l of bone marrow plus TGF- β 2 showed less bone and a higher level of fibrosis than the group with bone marrow plus activin. Although the alkaline phosphatase activity was elevated, it was less
5 than the level observed with bone marrow plus activin. These experimental results demonstrate that activin uniquely enhances bone formation with the absence of fibrosis when administered with bone marrow.

The present invention is shown and described
10 herein and was considered to be the most practical, and preferred embodiment. It is recognized, however, that departures may be made therefrom which are within the scope of the invention and that obvious modifications will occur to one skilled in the art upon reading this
15 disclosure.

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Claims

1. A composition for inducing bone growth and maturation in a subject comprising an osteogenically effective amount of activin in a pharmaceutically acceptable excipient.

2. The composition of claim 1, wherein said pharmaceutically acceptable excipient comprises a mixture of collagen and calcium phosphate mineral.

3. The composition of claim 1, further comprising an effective amount of BMP.

4. The composition of claim 3, wherein said BMP and said activin are present in a ratio of about 1:0.01 to about 1:100 by weight.

5. The composition of claim 3, wherein said BMP and said activin are present in a ratio of about 1:0.01 to about 1:1 by weight.

6. The composition of claim 1, which further comprises an effective amount of bone marrow.

7. The composition of claim 6, wherein said activin and said bone marrow are present in a ratio of about 0.001 μ g : 1 g to about 100 mg : 1 g by weight.

8. The composition of claim 1, further comprising

an effective amount of a BMP;
an effective amount of bone marrow, and
a pharmaceutically acceptable excipient.

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9. The composition of claim 8, wherein the pharmaceutically acceptable excipient comprises a mixture of collagen and calcium phosphate mineral.

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10. A method for inducing deposition and maturation of bone, which comprises administering an osteogenically effective amount of activin in a pharmaceutically acceptable excipient to a subject.

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11. The method of claim 10, wherein the activin in a pharmaceutically acceptable excipient is administered systemically to the subject.

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12. The method of claim 10, wherein the activin is administered once per day for about 1 to 14 days, preferably for about 3 to 10 days, and most preferably for about 7 days.

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13. The method of claim 10, wherein said subject has osteoporosis.

14. The method of claim 10, wherein said activin is administered locally.

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15. The method of claim 10, wherein said activin is administered in a dental or orthopedic implant.

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16. The method of claim 10, wherein the pharmaceutically acceptable excipient comprises a mixture of collagen and calcium phosphate mineral.

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17. The method of claim 10, which further comprises administering an effective amount of TGF- β to the subject.

5 18. The method of claim 17, wherein said activin and the TGF- β are administered simultaneously.

19. The method of claim 17, wherein said activin and said TGF- β are administered sequentially.

10

20. The method of claim 17, wherein said activin and said TGF- β are administered in a dental or orthopedic implant.

15

21. The method of claim 10, which further comprises administering an effective amount of a BMP to the subject.

20

22. The method of claim 21, wherein said activin and said BMPs are administered locally.

25

23. The method of claim 21, wherein said activin and said BMPs are administered in a ratio of about 1:0.01 to about 1:100 by weight.

30

24. The method of claim 21, wherein said activin and said BMPs are administered in a ratio of about 1:0.1 to about 1:1 by weight.

25. The method of claim 21, wherein said activin and said BMP administered systemically to the subject.

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26. The method of claim 21, wherein said activin and said BMP are administered once per day for about 1 to 14 days, preferably for about 3 to 10 days, and most preferably for about 7 days.

5

27. The method of claim 21, wherein said subject has osteoporosis.

28. The method of claim 21, wherein said
10 activin and said BMP are administered simultaneously.

29. The method of claim 21, wherein said activin and said BMP are administered sequentially.

15 30. The method of claim 21, wherein said activin and said BMP are administered in a dental or orthopedic implant.

31. The method of claim 21, which further
20 comprises administering an effective amount of TGF- β to the subject.

32. The method of claim 31, wherein said
25 activin, said BMP, and said TGF- β are administered in a dental or orthopedic implant.

33. The method of claim 10, which further
comprises administering an effective amount of bone
marrow to the subject.

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34. The method of claim 33, wherein said
activin and said bone marrow are administered once per
day for about 1 to 14 days, preferably for about 3 to 10
days, and most preferably for about 7 days.

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-34-

35. The method of claim 33 wherein said subject has osteoporosis.

5 36. The method of claim 33, wherein said composition is administered locally.

37. The method of claim 33, which further comprises administering an effective amount of TGF- β to
10 the subject.

38. The method of claim 33, wherein said activin and said bone marrow are present in a ratio of about 0.001 μ g : 1 g to about 100 mg : 1 g by weight.
15

39. The method of claim 33, wherein said bone marrow and said activin are administered in a composition comprising collagen and calcium phosphate mineral.

20 40. The method of claim 33, wherein said activin and said bone marrow are administered locally to an area of bone in need of treatment.

41. The method of claim 33, wherein said
25 activin and said bone marrow are administered simultaneously.

42. The method of claim 33, wherein said
30 activin and said bone marrow are administered sequentially.

43. The method of claim 33, wherein said
35 activin and said bone marrow are administered in a dental or orthopedic implant.

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44. A process for extracting and isolating activin from bone, comprising:

- 5 (a) extracting and isolating activin from demineralized bone powder,
- (b) fractionating the extract by gel filtration chromatography,
- (c) desalting the fraction containing TGF- β s and fractionating by cation exchange chromatography,
- 10 (d) fractionating the pool containing activin by RP-HPLC, using a linear acetonitrile gradient in TFA;
- (e) equilibrating the cation-exchange column with buffers; and
- (f) desalting the gradient using RP-HPLC in
- 15 TFA with a linear acetonitrile gradient.

45. A process for removing TGF- β contamination from an extract containing substantially pure activin, comprising performing a series of pH gradients on a

20 cation exchange column, wherein the preferred series of pH gradients are pH 4.6 to 6.7 and pH 6.7 to 9.0.

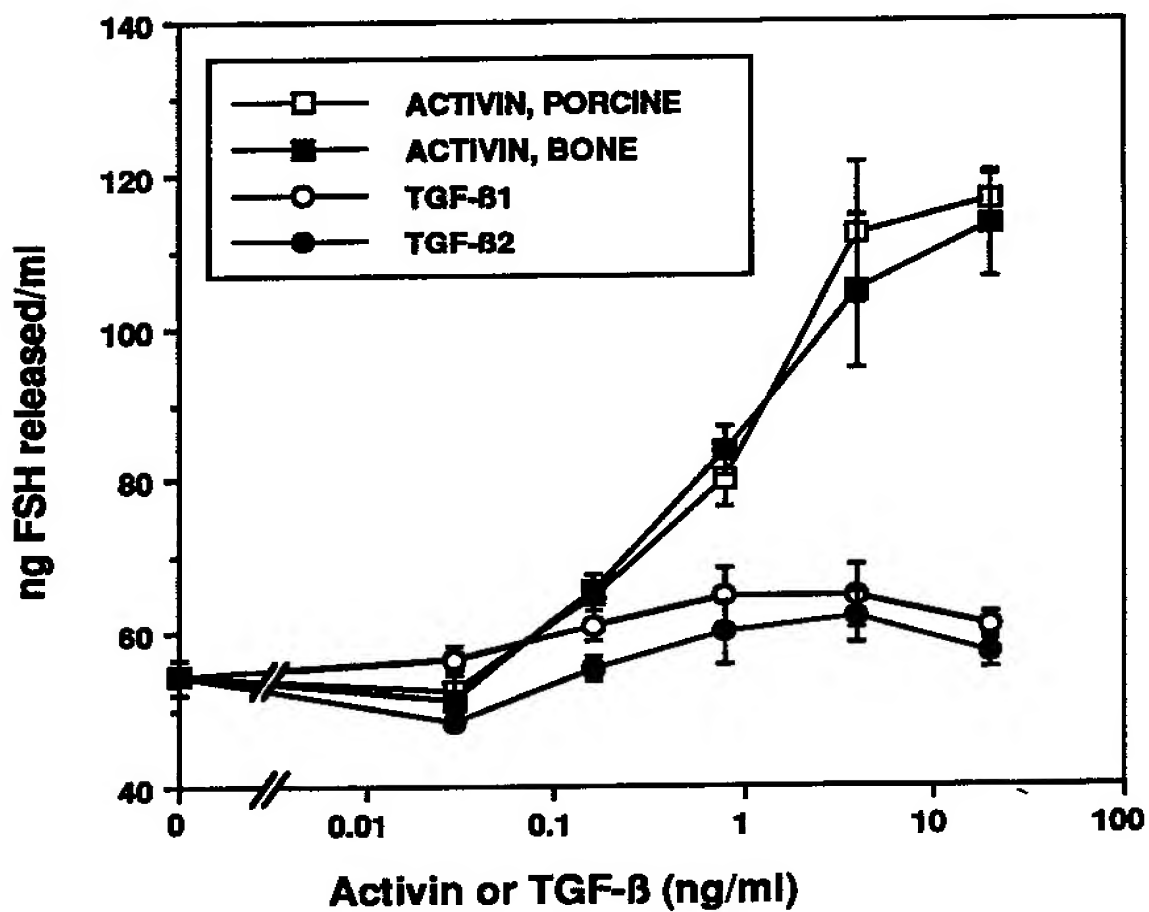
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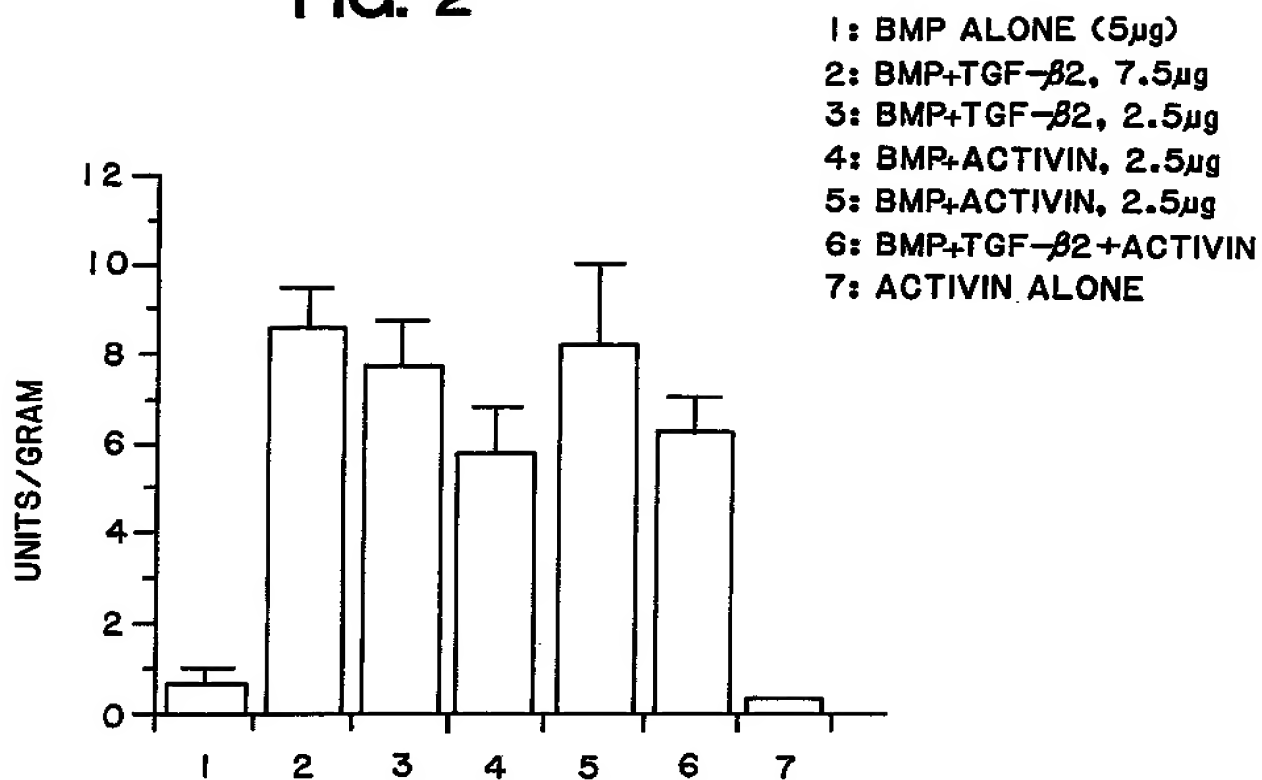
1 / 4

FIG. 1

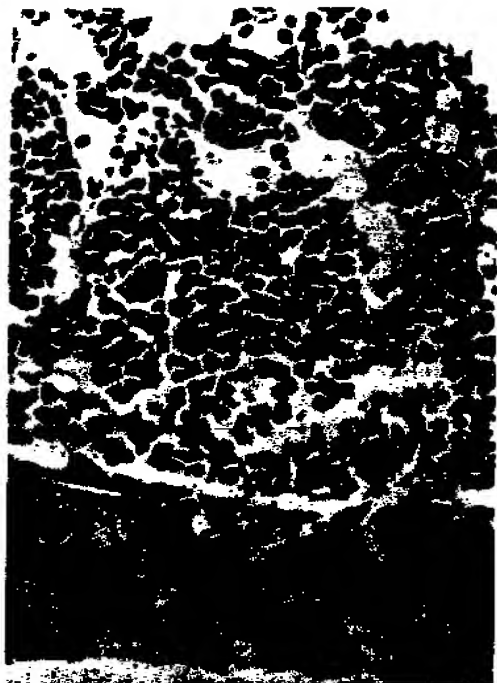


SUBSTITUTE SHEET

2 / 4

FIG. 2**SUBSTITUTE SHEET**

3 / 4



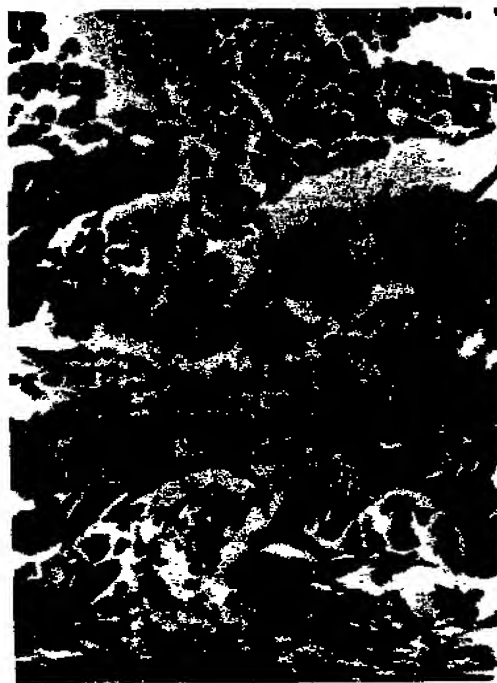
1 μ g activin/day

FIG. 3C



vehicle alone

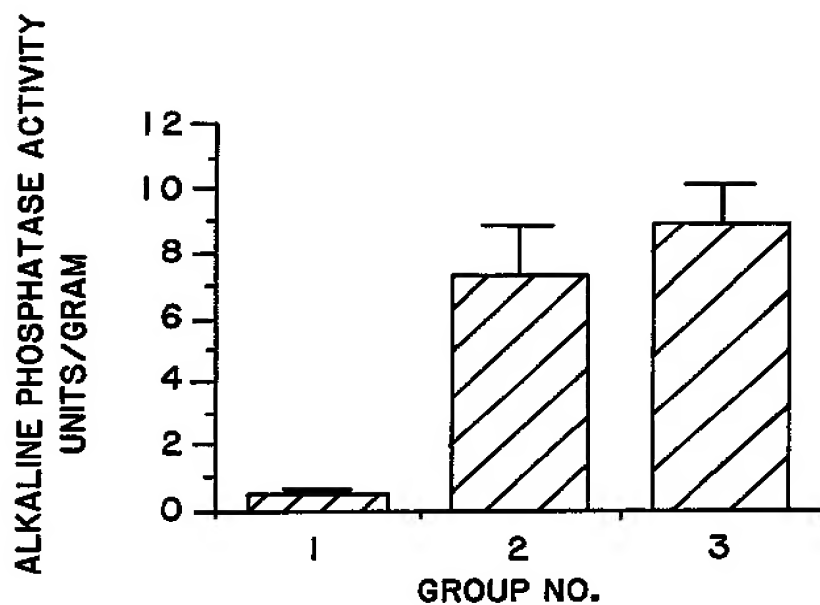
FIG. 3A



1 μ g TGF- β 1/day

FIG. 3B

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FIG. 4

GROUP 1: CERAMIC/COLLAGEN CARRIER ALONE

2: CERAMIC/COLLAGEN CARRIER+20 μ l BONE MARROW

3: CERAMIC /COLLAGEN CARRIER+20 μ l BONE MARROW
+ 5 μ g ACTIVIN

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01287

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 37/02; C07 K 3/20, 13/00 US CL : 514/2, 8, 12, 21; 530/399, 412, 416																	
II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched⁴</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; padding: 5px;">Classification System</th> <th style="border: 1px solid black; padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; text-align: center;">U.S.</td> <td style="border: 1px solid black; padding: 5px;">514/2, 8, 12, 21; 530/399, 412, 416</td> </tr> </table>			Classification System	Classification Symbols	U.S.	514/2, 8, 12, 21; 530/399, 412, 416											
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Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵ Cas On-Line and APS																	
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category*</th> <th style="width: 70%; padding: 5px;">Citation of Document,¹⁶ with indication, where appropriate, of the relevant passages¹⁷</th> <th style="width: 20%; padding: 5px;">Relevant to Claim No. ¹⁸</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">Nature, Volume 321, issued 19 June 1986, Wale et al. "Purification and Characterization of an FSH Releasing Protein from Porcine Ovarian Follicular Fluid", pages 776-779, see Table 1 on page 778 to column 2, paragraph 2 of page 779.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1,44,45</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">The Journal of Biological Chemistry, Volume 261, No. 13, issued May 1986, S.M. Seyedin et al., "Cartilage-inducing Factor A", pages 5693-5695, see abstract.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-43</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">J. Cell Biol. Volume 110, issued June 1990, M. Joyce et al. "Transforming Growth Factor-B and the Initiation of Chondrogenesis and Osteogenesis in the Rat Femur", pages 2195-2207, see abstract.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-43</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Experimental Hematology, Volume 16, No. 6, issued July 1988, H. E. Broxmeyer et al. "Purified Recombinant Human Activin and Inhibin Respectively Enhance and Suppress Proliferation of Human Bone Marrow Multipotential and Erythroid, but not Granulocyte-Macrophage, Progenitor Cells Through Actions Mediated by Monocytes and T-Lymphocytes in The Absence or Presence of Serum", page 474, see abstract No. 62.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-43</td> </tr> </tbody> </table>			Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	Y	Nature, Volume 321, issued 19 June 1986, Wale et al. "Purification and Characterization of an FSH Releasing Protein from Porcine Ovarian Follicular Fluid", pages 776-779, see Table 1 on page 778 to column 2, paragraph 2 of page 779.	1,44,45	Y	The Journal of Biological Chemistry, Volume 261, No. 13, issued May 1986, S.M. Seyedin et al., "Cartilage-inducing Factor A", pages 5693-5695, see abstract.	1-43	Y	J. Cell Biol. Volume 110, issued June 1990, M. Joyce et al. "Transforming Growth Factor-B and the Initiation of Chondrogenesis and Osteogenesis in the Rat Femur", pages 2195-2207, see abstract.	1-43	A	Experimental Hematology, Volume 16, No. 6, issued July 1988, H. E. Broxmeyer et al. "Purified Recombinant Human Activin and Inhibin Respectively Enhance and Suppress Proliferation of Human Bone Marrow Multipotential and Erythroid, but not Granulocyte-Macrophage, Progenitor Cells Through Actions Mediated by Monocytes and T-Lymphocytes in The Absence or Presence of Serum", page 474, see abstract No. 62.	1-43
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Y	The Journal of Biological Chemistry, Volume 261, No. 13, issued May 1986, S.M. Seyedin et al., "Cartilage-inducing Factor A", pages 5693-5695, see abstract.	1-43															
Y	J. Cell Biol. Volume 110, issued June 1990, M. Joyce et al. "Transforming Growth Factor-B and the Initiation of Chondrogenesis and Osteogenesis in the Rat Femur", pages 2195-2207, see abstract.	1-43															
A	Experimental Hematology, Volume 16, No. 6, issued July 1988, H. E. Broxmeyer et al. "Purified Recombinant Human Activin and Inhibin Respectively Enhance and Suppress Proliferation of Human Bone Marrow Multipotential and Erythroid, but not Granulocyte-Macrophage, Progenitor Cells Through Actions Mediated by Monocytes and T-Lymphocytes in The Absence or Presence of Serum", page 474, see abstract No. 62.	1-43															
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	J. Biol. Chem., Volume 264, no. 32, H. Bentz et al., issued 05 December 1989 "Purification and Characterization of a Unique Osteoinductive Factor", pages 20805-20810, see abstract.	44,45
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V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. ☐ Claim numbers, because they relate to subject matter (1) not required to be searched by this Authority, namely:
2. ☐ Claim numbers, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:
3. ☐ Claim numbers, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This international Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.